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Inhibition of cytochrome P450 activity enhances the systemic availability of triclabendazole metabolites in sheep

G. VIRKEL*,†
A. LIFSCHITZ*,†
J. SALLOVITZ*,‡
M. BALLENT*,†
S. SCARCELLA* &
C. LANUSSE*,†

**Laboratorio de Farmacología, Dpto. de Fisiopatología, Facultad de Ciencias Veterinarias, UNCPBA, Tandil, Buenos Aires, Argentina; †Consejo Nacional de Investigaciones Científicas y Tecnológicas, Buenos Aires, Argentina; ‡Comisión de Investigaciones Científicas, Buenos Aires, Argentina*

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Understanding the disposition kinetics and the pattern of metabolism is critical to optimise the flukicidal activity of triclabendazole (TCBZ) in ruminants. TCBZ is metabolised by both flavin-monooxygenase (FMO) and cytochrome P450 (P450) in the liver. Interference with these metabolic pathways may be useful to increase the systemic availabilities of TCBZ metabolites, which may improve the efficacy against *Fasciola hepatica*. The plasma disposition of TCBZ metabolites was evaluated following TCBZ co-administration with FMO [methimazole (MTZ)] and P450 [piperonyl butoxyde (PB) and ketoconazole (KTZ)] inhibitors in sheep. Twenty (20) healthy Corriedale x Merino weaned female lambs were randomly allocated into four experimental groups. Animals of each group were treated as follow: Group A, TCBZ alone (5 mg/kg, IV route); Group B, TCBZ (5 mg/kg, IV) + MTZ (3 mg/kg, IV); Group C, TCBZ (5 mg/kg, IV) + PB (30 mg/kg, IV) and Group D, TCBZ (5 mg/kg, IV) + KTZ (10 mg/kg, orally). Blood samples were taken over 240 h post-treatment and analysed by HPLC. TCBZ sulphoxide and sulphone were the main metabolites recovered in plasma. MTZ did not affect TCBZ disposition kinetics. TCBZ sulphoxide Cmax values were significantly increased ($P < 0.05$) after the TCBZ + PB (62%) and TCBZ + KTZ (37%) treatments compared to those measured in the TCBZ alone treatment. TCBZ sulphoxide plasma AUCs were higher ($P < 0.05$) in the presence of both PB (99%) and KTZ (41%). Inhibition of TCBZ P450-mediated oxidation in the liver accounted for the increased systemic availability of its active metabolite TCBZ sulphoxide. This work contributes to the search of different strategies to improve the use of this flukicidal drug in ruminants.

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Dr Guillermo Virkel, Laboratorio de Farmacología, Facultad de Ciencias Veterinarias, UNCPBA, (7000) Tandil, Buenos Aires, Argentina. E-mail: gvirkel@vet.unicen.edu.ar

INTRODUCTION

Triclabendazole [2–methylthio-5(6)–chloro-6(5)-(2′,3′-dichloro) phenoxy-benzimidazole (TCBZ)] is an halogenated benzimidazole (BZD) anthelmintic used worldwide to control immature and adult stages of the liver fluke *Fasciola hepatica* (Boray *et al.*, 1983). The plasma pharmacokinetic profile of TCBZ has been investigated in ruminant species, including cattle (Bogan *et al.*, 1988), sheep (Hennessy *et al.*, 1987; Bogan *et al.*, 1988) and goats (Bogan *et al.*, 1988). In sheep, the kinetics of TCBZ disposition in plasma and bile has been thoroughly described (Hennessy *et al.*, 1987). TCBZ parent drug was not detected in plasma after its oral administration to weaned lambs, indicating its complete removal from portal blood by the liver after intestinal absorption. Overall, TCBZ was oxidised to form its primary plasma metabolites, i.e., triclabendazole sulphoxide (TCBZSO) and triclabendazole sulphone ($TCBZSO_2$). Extremely low concentrations of TCBZ parent compound were detected in bile, whereas TCBZSO, $TCBZSO_2$ and the hydroxy-derivatives were the major biliary metabolites recovered in TCBZ-treated sheep in their unconjugated and conjugated forms (Hennessy *et al.*, 1987). TCBZ metabolites are strongly bound to plasma proteins (≥90%), which may account for their long residence time in the animal's body (Hennessy *et al.*, 1987).

Sulphoxidation and sulphonation appear to be the main metabolic reactions involved in TCBZ hepatic biotransformation

in sheep. A recent *in vitro* investigation carried out in our laboratory showed that both mixed function oxidases, flavin monooxygenase (FMO) and cytochrome P450 (P450), are involved in such metabolic reactions in sheep liver (Virkel *et al.*, 2006). We also investigated the effect of well-known enzymatic inhibitors on TCBZ metabolism by sheep liver microsomes. Therefore, TCBZ sulphoxidative metabolism was reduced in the presence of the FMO-inhibitor methimazole (MTZ), and also when the anthelmintic molecule was incubated in the presence of the P450 inhibitor piperonyl butoxide (PB). Moreover, the rate of TCBZSO conversion into $TCBZSO_2$ was also significantly inhibited by either metabolic inhibitor. Inhibition of TCBZ sulphonation was also observed in the presence of other P450 inhibitors such as ketoconazole (KTZ) and erythromycin. The pharmacokinetic study described here follows up our previous work and it was designed to ascertain whether or not such *in vitro* metabolic interactions might be observed under *in vivo* conditions. Inhibitors of FMO or P450 enzymatic systems have been shown to interfere with the biotransformation of other BZD anthelmintics either *in vivo* or *in vitro* (Lanusse & Prichard, 1993). Thus, interference with the liver oxidative metabolism has resulted in pronounced modifications to the pharmacokinetic behaviour of active BZD parent drugs or their active metabolites, which may improve the clinical efficacy of these anthelmintics. The purpose of the work described here was to evaluate the plasma disposition kinetics of TCBZ metabolites following TCBZ co-administration with known inhibitors of the FMO and P450 enzymatic systems in sheep.

MATERIALS AND METHODS

Chemicals

Reference standards (99% pure) of TCBZ and its –sulpho metabolites (TCBZSO and $TCBZSO_2$) were provided by Novartis Animal Health (Basel, Switzerland). Stock solutions (1000 μg/mL) of each analyte were prepared in methanol (Baker Inc., Phillipsburg, USA). Methimazole (MTZ) was a generous gift of Gador Argentina S.A. (an injectable solution of 50 mg/mL was prepared in sterile deionised water). Piperonyl butoxide (PB) was purchased from Sigma-Aldrich Chemical Company (St. Louis, USA.). An oral formulation of ketoconazole (KTZ) was obtained from J'anvier (Buenos Aires, ARGENTINA). The solvents used for chemical extraction and chromatographic analyses were HPLC grade (Baker Inc., Phillipsburg, USA).

Experimental animals and treatments

Twenty (20) healthy Corriedale x Merino weaned female lambs (18.6 ± 4.2 kg), identified with ear tags, were randomly allocated into four (4) treatment groups of five animals each and placed in indoor facilities during the trials. Sheep were fed with high quality concentrated food and had free access to water. Animals of each group were treated as follow: Group A, TCBZ alone (5 mg/kg, IV route); Group B, TCBZ (5 mg/kg, IV) + MTZ (3 mg/kg, IV); Group C, TCBZ (5 mg/kg, IV) + PB (30 mg/kg, IV) and Group D, TCBZ (5 mg/kg, IV) + KTZ (10 mg/kg, orally). Intravenous treatments were done in the right jugular vein. TCBZ was formulated as a 15% (w/v) solution in propylene glycol/dimethyl sulphoxide (90/10). Animal procedures and management protocols were carried out according to the Animal Welfare Policy (Academic Council Resolution 087/02) of the Faculty of Veterinary Medicine, Universidad Nacional del Centro de la Provincia de Buenos Aires (UNCPBA), Tandil, Argentina *(http://www.vet.unicen.edu.ar)*.

Sample collection

Blood samples were taken from the left jugular vein into heparinised vacutainer tubes prior to and at 5, 10, 15, 30, 45 min, 1, 2, 4, 8, 12, 24, 36, 48, 76, 96, 120, 144, 192 and 240 h post-treatment. The collected blood samples were centrifuged at 1000 ***g*** during 15 min and plasma stored at −20 °C until the time of analysis.

Analytical procedures

Sample clean-up/extraction

Aliquots of the collected plasma (0.5 mL) were supplemented with oxibendazole (OBZ) (as internal standard, 99.2% pure, 1 μg/40 μL methanol) and then mixed with 2 mL acetonitrile. The samples were shaken for 20 min in a multivortex and then centrifuged at 2500 ***g*** for 15 min. Supernatants were evaporated to dryness using an Automatic Environmental Speed Vac System (Savant, Holbrook, USA). The dry residue was dissolved in 300 μL mobile phase and 50 μL were injected into the high performance liquid chromatography (HPLC) system.

Drug and metabolite analysis

Samples were analysed for TCBZ and its metabolites. Fifty microlitres (50 μL) of each extracted sample were injected through an autosampler (Shimadzu SIL 10 A Automatic Sample Injector) into a Shimadzu 10 A HPLC system (Shimadzu Corporation, Kyoto, Japan) fitted with a Kromasil C_{18} (5 μm, 250 mm × 4.60 mm) reverse-phase column (Eka Chemicals, USA) and a UV detector (Shimadzu, SPD-10A UV detector) reading at 292 nm. The mobile phase was composed of acetonitrile/ammonium acetate (0.025 M, pH 6.6) pumped as an elution gradient (0–5 min: 52/48; 6–12 min: 64/36; 13–16 min: 52/48). The analytes were identified with the retention times of 97–99% pure reference standards. Under these chromatographic conditions the retention times were 4.1 min (OBZ, used as internal standard), 6.5 min ($TCBZSO_2$), 8.3 min (TCBZSO) and 14.1 min (TCBZ). Chromatographic peak areas of each molecule were measured using the integrator software (Class LC 10, Shimadzu Corporation, Kyoto, Japan) of the HPLC system.

Drug/metabolites quantification

Validation of the analytical procedures for extraction and quantification of TCBZ and its metabolites was performed before starting the analysis of the experimental samples. Known amounts of each analyte (0.05–10.0 μg/mL) were added to

aliquots of plasma collected from untreated sheep. Fortified samples were extracted and analysed by HPLC (triplicate determinations) to obtain calibration curves and percentages of recovery. Calibration curves were analysed using the least squares linear regression analysis (Instat 3.00, Graph Pad Software, Inc., San Diego, USA) of HPLC peak area ratios of analytes/IS and nominal concentrations of spiked samples. Correlation coefficients (r) for the different analytes ranged between 0.995 and 0.999. A lack-of-fit test was also carried out to confirm the linearity of the regression line of each analyte. The concentrations in the experimental samples were determined following interpolation using the standard curves. Absolute recoveries were established by comparison of the detector responses (peak areas) obtained for spiked plasma samples and those of direct standards prepared in mobile phase (concentration range between 0.05 and 2 μg/mL). Drug/metabolite absolute recoveries from plasma were: 82–87% ($TCZSO_2$), 86–91% (TCBZSO) and 76–87% (TCBZ). The inter-assay precision of the extraction and chromatographic procedures were estimated by processing replicate aliquots ($n = 4$) of pooled sheep plasma samples containing known concentrations of each molecule (0.5, 1 and 2 μg/mL) on different working days. Inter-assay precision coefficients of variation (CVs) were <15% and relative error (accuracy) values were <20%.

Pharmacokinetic and statistical analyses of the data

The concentration vs. time curves for TCBZ and its metabolites in plasma for each individual animal after the different treatments were fitted with PK Solution 2.0 (Summit research services, CO, USA). The data points generated for TCBZ in plasma after its i.v. administration were best-fitted to a two-compartment open model (Notari, 1987):

$$Cp = Ae^{-\alpha t} - Be^{-\beta t}$$

where A and B are the primary and secondary disposition intercepts; α and β are the primary and secondary disposition rate constants; and Cp was the plasma concentration of TCBZ at time t. The distribution and elimination half-lives were calculated as ln 2 divided by the rate constants. The estimated plasma concentration of TCBZ parent drug at zero time ($C_{p(0)}$) after its i.v. administration was the sum of the extrapolated zero-time concentrations of the coefficient A and B. Total body clearance (Cl_B) was calculated by:

$$Cl_B = Dose/AUC$$

The volume of distribution ($V_{D\ (area)}$) was estimated by the following equation:

$$V_{D(area)} = Dose/(AUC)(\beta)$$

The following equation (Notari, 1987) was used to describe the biexponential concentration-time curves for TCBZ metabolites (sulphoxide and sulphone) after the intravenous administration of TCBZ:

$$Cp = Be^{-\beta t} - Be^{-kt}$$

where Cp = concentration in plasma at time t after administration (μg/mL); B = concentration at time zero extrapolated from the elimination phase (μg/mL); e = base of the natural logarithm; β = terminal slope (h^{-1}); and k is the slope obtained by feathering which represents the first order metabolite formation rate constant (k_{for}) (h^{-1}). The elimination half-life ($t_{1/2}\beta$) and metabolite formation half-lives ($t_{1/2for}$) were calculated as $\ln 2/\beta$ and ln2/k, respectively. The peak concentration (Cmax) and time to peak concentration (Tmax) were displayed from the plotted concentration-time curve of each analyte. The area under the concentration time curve (AUC) was calculated by the trapezoidal rule (Gibaldi & Perrier, 1982) and further extrapolated to infinity by dividing the last experimental concentration by the terminal slope (β). The mean residence time (MRT) was determined as AUMC/AUC (Perrier & Mayersohn, 1982) where AUMC is the area under the curve of the product of time and the plasma drug concentration vs. time (Gibaldi & Perrier, 1982) and AUC is as defined above.

Drug concentrations and all the estimated pharmacokinetic parameters are reported as mean ± SD. A normality test was performed for testing if the data are sampled from populations that follow Gaussian distributions. This assumption was tested using the Kolmogorov and Smirnov method. Mean pharmacokinetic parameters for different experimental groups were statistically compared using ANOVA. Where significant overall differences ($P < 0.05$) were observed, further analysis among TCBZ treatments was performed using Bonferroni Multiple Comparisons Test. The assumption that the data, obtained after all treatments, have the same variance was also assessed.

RESULTS

Triclabendazole parent drug displayed a biexponential plasma concentration vs. time curve following its IV administration to sheep. TCBZSO and $TCBZSO_2$ were the metabolites detected in the bloodstream. The mean (±SD) plasma concentration profiles for TCBZ and its sulpho-derivatives after the IV administration of TCBZ parent drug to sheep (control group) is shown in Fig. 1. TCBZ was rapidly depleted from plasma and was detected in the bloodstream between 5 min and 12 h post-treatment. Similarly, TCBZ metabolites appeared rapidly in the bloodstream (TCBZSO = 5 min, $TCBZSO_2$ = 10 min), reaching peak concentrations at 2.8 h (TCBZSO) and 21.6 h ($TCBZSO_2$) post-treatment.

The kinetic parameters characterising the plasma disposition of TCBZ after its IV administration either alone (control group) or co-administered with the different metabolic inhibitors are compared in Table 1. As stated above, the parent drug was rapidly depleted from plasma and was detected up to 12 h postadministration of TCBZ alone or co-administered with each

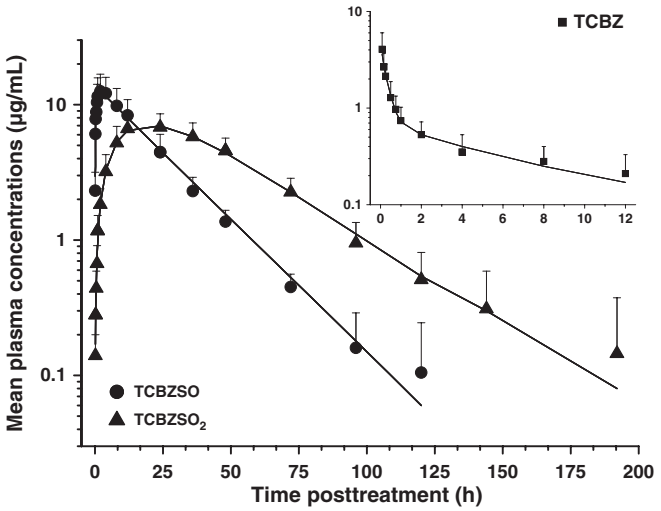

Fig. 1. Mean (±SD) plasma concentrations (µg/mL) of triclabendazole sulphoxide (TCBZSO) and sulphone ($TCBZSO_2$) metabolites after the intravenous administration (5 mg/kg) of an injectable formulation of triclabendazole (TCBZ) to sheep ($n = 5$). The inserted graph shows the plasma profile of TCBZ parent drug.

metabolic inhibitor. Additionally, no statistical differences were observed among the pharmacokinetic parameters describing the disposition kinetics of the parent drug after the different experimental treatments.

The comparative TCBZSO plasma concentration profiles obtained after the administration of TCBZ either alone or co-administered with the different metabolic inhibitors is shown in Fig. 2. Following all experimental treatments, TCBZSO was detected in plasma from 5 min and up to 120 h postadministration of TCBZ. Mean pharmacokinetic parameters for TCBZSO obtained after all experimental treatments are shown in Table 2. MTZ did not modify the pharmacokinetic behaviour of TCBZSO under the experimental conditions described in this work. On the other hand, TCBZSO plasma availability (measured as AUC) was higher when both PB (99%, $P < 0.01$) and KTZ (40.7%, $P < 0.05$) were individually co-administered with TCBZ compared to that observed when the flukicidal drug was administered alone. In addition, TCBZSO peak plasma concentrations (Cmax) increased after the administration of TCBZ + PB (62%, $P < 0.01$) and TCBZ + KTZ (37%, $P < 0.05$) compared to that measured following the administration of TCBZ alone.

Triclabendazole sulphone was detected in plasma from 10 min to 144 h after administration of TCBZ alone or co-administered with each metabolic inhibitor (Fig. 3). Table 3 compares the pharmacokinetic parameters for $TCBZSO_2$ obtained after all experimental treatments. MTZ and KTZ did not modify the disposition kinetics of $TCBZSO_2$. Conversely, $TCBZSO_2$ AUC and Cmax values were enhanced following the TCBZ + PB administration compared to those measured when TCBZ was administered alone.

Figure 4 shows the partial TCBZSO AUC (0–36 h)/$TCBZSO_2$ AUC (0–36 h) ratios obtained following all TCBZ experimental treatments assayed. TCBZSO/$TCBZSO_2$ AUC (0–36 h) ratios were higher following TCBZ + PB (40%) and TCBZ + KTZ (19%) treatments.

DISCUSSION

Benzimidazole anthelmintics require extensive hepatic oxidative metabolism to achieve sufficient polarity for excretion (Hennessy *et al.*, 1993). As a common pattern among different BZDs, the parent drug is short lived and metabolic products predominate in most target tissues, as well as within parasites recovered from BZD-treated animals (Alvarez *et al.*, 1999, 2000; Cristofol *et al.*, 2001). Sulphur-containing BZD-methylcarbamates are metabolised in the liver into their respective sulphoxide, sulphone and hydroxylated metabolites (Gottschall *et al.*, 1990). In terms of parasite uptake/accumulation (Alvarez *et al.*, 1999, 2000) and mode of action (binding to tubulin) (Lubega & Prichard, 1991), the parent thioethers (such as TCBZ) are more efficient than their respective oxidised metabolites. Consequently, their metabolic pattern and the resultant pharmacokinetic behaviour are relevant in the attainment of high and sustained concentrations of pharmacologically active drug/metabolites at the target parasite (Lanusse & Prichard, 1993).

A biexponential plasma concentration vs. time curve characterised the disposition kinetics of TCBZ following its IV administration. The drug was rapidly depleted from the systemic circulation, being undetectable beyond 12 h post-treatment.

Table 1. Comparative disposition kinetics of triclabendazole (TCBZ) parent drug after its intravenous administration (5 mg/kg) either alone (control group) or co-administered with the metabolic inhibitors methimazole (MTZ), piperonyl butoxide (PB) and ketoconazole (KTZ)

Pharmacokinetic parameters	TCBZ control	TCBZ + MTZ	TCBZ + PB	TCBZ + KTZ
$C_{p(0)}$ (µg/mL)	4.44 ± 2.41	4.11 ± 1.48	5.97 ± 1.02	4.91 ± 1.78
t½α (h)	0.27 ± 0.18	0.23 ± 0.07	0.22 ± 0.07	0.19 ± 0.04
t½β (h)	7.24 ± 4.06	7.50 ± 5.90	8.61 ± 2.95	5.42 ± 3.92
AUC $_{0–12\ h}$ (µg·h/mL)	5.48 ± 1.90	4.74 ± 0.79	6.91 ± 2.54	4.87 ± 1.16
MRT (h)	3.70 ± 0.77	3.34 ± 0.59	3.51 ± 0.78	3.10 ± 0.83
$V_{D\ (area)}$ L/kg	7.69 ± 5.59	10.3 ± 6.60	7.05 ± 2.87	6.88 ± 2.94
Cl_B (mL/h·kg)	701 ± 393	660 ± 320	569.5 ± 310.1	707.9 ± 472.9

$C_{p(0)}$, estimated initial (at time zero) drug concentration in plasma; t½α, distribution half-life; t½β, elimination half-life; AUC $_{0–12\ h}$, area under concentration-time curve from time 0 to 12 h after drug treatment; MRT, mean residence time; $V_{D\ (area)}$, apparent volume of distribution based on AUC; Cl_B: body clearance.

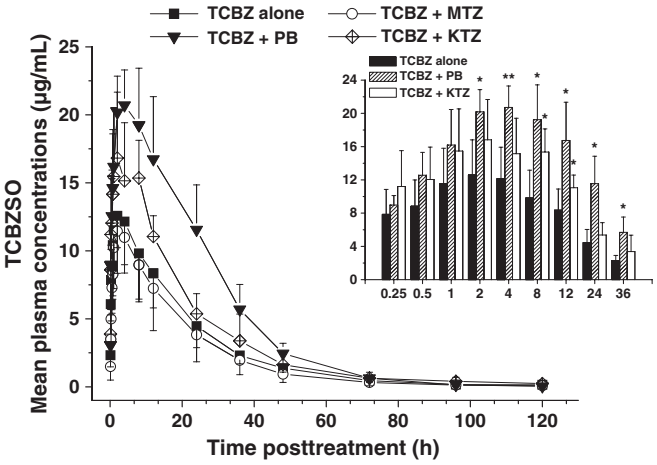

Fig. 2. Comparative mean (±SD) plasma concentration profiles (μg/mL) of triclabendazole sulphoxide (TCBZSO) in sheep after the intravenous administration of triclabendazole (TCBZ) parent drug (5 mg/kg) either alone or co-administered with different metabolic inhibitors: methimazole (MTZ), piperonyl butoxide (PB) and ketoconazole (KTZ). The inserted graph shows TCBZSO plasma concentrations from 0.25 to 36 h postadministration of TCBZ alone or co-administered with PB or KTZ. Values are significantly different from the TCBZ alone treatment at (**) $P < 0.01$ and (*) $P < 0.05$.

Mean TCBZ t½α values ranged between 0.19 and 0.27 h and characterised its distribution phase. The fast disappearance of TCBZ from plasma during this initial phase confirms its extensive oxidation in the liver and in extra-hepatic tissues, but also indicates the wide tissue distribution of the anthelmintic drug (V_D values much higher than 1 L/kg). In addition, this pattern of TCBZ depletion from the systemic circulation may indicate that the drug is sequestered in the liver, as it was previously suggested for albendazole (Hennessy *et al.*, 1989). Therefore, TCBZ concentration in the liver tissue, even when the drug is administered by an enteral route, may be relevant for its activity against migrating immature fluke stages.

The rapid disappearance of TCBZ from the bloodstream and the early appearance of its sulphoxide and sulphone metabolites confirm the rapid hepatic and extra-hepatic biotransformation of the parent drug. TCBZ metabolites bind strongly (≥90%) to plasma proteins, specifically albumin (Hennessy *et al.*, 1987). The long persistence of TCBZ metabolites in the bloodstream (over 120 h post-treatment) and their high plasma concentrations, compared to other BZD compounds, correlate with their extensive plasma protein binding. This enhanced plasma profile of TCBZ metabolites may contribute to the drug flukicidal activity based on a high and prolonged drug exposure of blood-feeding adult flukes.

As previously shown for the most commonly used BZDs, two sequential oxidative steps (sulphoxidation and sulphonation) appear to be the main metabolic reactions involved in TCBZ hepatic biotransformation in sheep. The sulphoxide metabolite may be reduced back to its parent thioether by ruminal microflora (Virkel *et al.*, 2006), but also may undergo a second, slower and irreversible oxidative step that forms the sulphone derivative. *In vitro* investigations carried out in our laboratory showed that both FMO and P450 are involved in TCBZ biotransformation in sheep liver (Virkel *et al.*, 2006). While the FMO system was found as the main enzymatic pathway involved in the sulphoxidation of TCBZ (yielding ~77% of TCBZSO production), both enzymatic systems participate in a similar proportion in the sulphonation reaction to form the sulphone metabolite. Studies on *ex vivo* drug diffusion into TCBZ-susceptible *Fasciola hepatica*, demonstrated that TCBZ, TCBZSO and $TCBZSO_2$ have the capability to penetrate the fluke's tegument (Alvarez *et al.*, 2004). The parent TCBZ and its sulphoxide and sulphone metabolites showed a similar ability to penetrate into the trematode parasite. However, the diffusion of the hydroxy-derivatives into the fluke was lower than that observed for the sulpho-containing TCBZ derivatives (TCBZSO and $TCBZSO_2$). Although the intrinsic mechanism of action of TCBZ and its metabolites remains to be fully understood (Fairweather, 2005; Brennan *et al.*, 2007), TCBZ–sulpho and –hydroxy derivatives are supposed to be less potent than the parent drug. Therefore, both sequential oxidative steps may give rise to a considerable reduction in their anthelmintic efficacy.

In vivo interference with the liver FMO-mediated and/or P450-mediated metabolism has resulted in pronounced modifications to the pharmacokinetic behaviour of anthelmintically active BZD metabolites. It has been shown that co-administration of BZDs with known metabolic inhibitors enhances the plasma availabilities of the parent drug and/or their metabolites. Thus, the enhanced plasma levels and/or extended presence of active moieties may prolong the plasma-tissue recycling process and

Table 2. Comparative pharmacokinetic parameters for triclabendazole sulphoxide (TCBZSO) obtained after administration of triclabendazole (TCBZ) parent drug (5 mg/kg) either alone or co-administered with the metabolic inhibitors methimazole (MTZ), piperonyl butoxide (PB) and ketoconazole (KTZ)

Pharmacokinetic parameters	TCBZ control	TCBZ + MTZ	TCBZ + PB	TCBZ + KTZ
$t_{½for}$ (h)	0.29 ± 0.06	0.51 ± 0.23*	0.59 ± 0.18*	0.29 ± 0.12
Cmax (μg/mL)	12.9 ± 4.45	12.0 ± 1.10	20.9 ± 2.75**	17.7 ± 3.69*
Tmax (h)	2.80 ± 1.10	2.60 ± 1.34	5.00 ± 2.00	4.00 ± 3.67
t½β (h)	15.6 ± 1.77	12.2 ± 4.27	17.8 ± 2.00	17.4 ± 6.81
$AUC_{0–120\ h}$ (μg·h/mL)	297.6 ± 76.0	253.5 ± 96.1	592.5 ± 145.2**	418.7 ± 66.4*
MRT (h)	21.3 ± 4.05	17.9 ± 5.38	17.6 ± 1.01	26.1 ± 15.0

Data are expressed as mean ± SD ($n = 5$ animals).
Cmax, peak plasma concentration; Tmax, time to peak plasma concentration; $t_{½for}$, metabolite formation half-life; t½β, elimination half-life; $AUC_{0–120\ h}$, area under concentration-time curve from time 0 to 120 h after drug treatment; MRT, mean residence time. Values are significantly different from the TCBZ alone treatment at (*) $P < 0.05$a and $P < 0.01$.

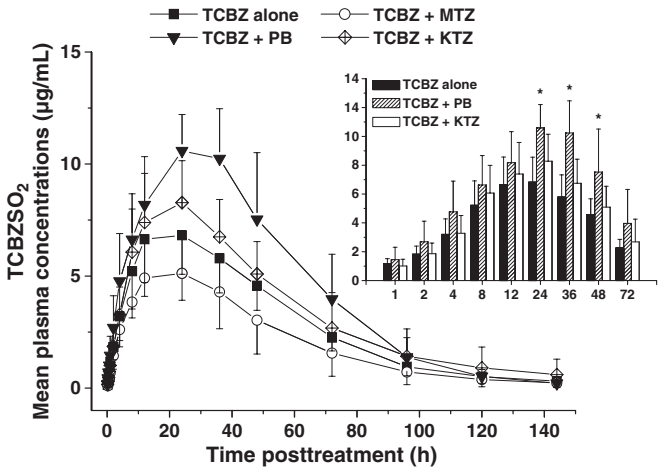

Fig. 3. Comparative mean (±SD) plasma concentration profiles (μg/mL) of triclabendazole sulphone ($TCBZSO_2$) in sheep after the intravenous administration of triclabendazole (TCBZ) parent drug (5 mg/kg) either alone or co-administered with different metabolic inhibitors: methimazole (MTZ), piperonyl butoxide (PB) and ketoconazole (KTZ). The inserted graph shows $TCBZSO_2$ plasma concentrations from 1 to 72 h postadministration of TCBZ alone or co-administered with PB or KTZ. Values are significantly different from the TCBZ alone treatment at (*) $P < 0.05$.

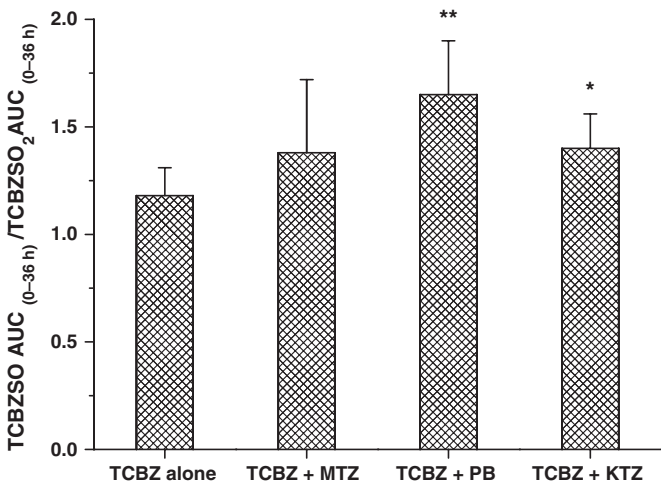

Fig. 4. Mean (±SD) ratios between the partial AUCs $_{(0–36\ h)}$ of TCBZSO and $TCBZSO_2$. MTZ, methimazole; PB, piperonyl butoxide; KTZ, ketoconazole. Values are significantly different from TCBZ control treatment at (**) $P < 0.01$ and (*) $P < 0.05$.

increase their concentrations at the most important tissues of parasite location (Lanusse & Prichard, 1993). This phenomenon may assure a significant parasite exposure to the drug and improved clinical efficacy for the administered compound as a consequence of enhanced active drug/metabolite concentrations being presented to the parasite for extended time periods (Lanusse & Prichard, 1993). In sheep, co-administration of oxfendazole with parbendazole (Hennessy *et al.*, 1992), MTZ (Lanusse *et al.*, 1995) or PB (Sánchez *et al.*, 2002) increased the concentrations of the active moieties (fenbendazole and oxfendazole itself) in the systemic circulation. Besides, MTZ and metyrapone, a potent inhibitor of the P450 system, improved the plasma availabilities of albendazole metabolites following the administration of the pro-BZD netobimin to sheep (Lanusse & Prichard, 1991, 1992). More than 3-fold increments in the AUC values of fenbendazole and its sulphoxide metabolite (oxfendazole) were observed following fenbendazole + PB administration to sheep and goats (Benchaoui & McKellar, 1996). Moreover, the concomitant administration of fenbendazole with PB markedly improved the efficacy against BZD-resistant strains of *Ostertagia circumcincta* and *Haemonchus contortus* in sheep (Benchaoui & McKellar, 1996). Co-administration of MTZ with netobimin or albendazole also increased the efficacy against arrested larvae of *Ostertagia ostertagi* and against total adult gastrointestinal nematodes in naturally infected cattle (Lanusse & Prichard, 1993). All these previous results clearly demonstrate the practical relevance of the interference with the liver oxidative metabolism, which may represent a useful tool to increase the antiparasitic efficacy of BZD anthelmintics in ruminants.

In the current work, TCBZ was administered by the IV route instead of the commonly used oral or intraruminal routes. Several factors, such as the dissolution rate of the formulation, the extent of drug adsorption to the particulate digesta material, the gastrointestinal transit time, the oesophageal grove closure and/or the metabolic activity of the ruminal microflora have been shown to affect drastically the rate and the extent of absorption (and the systemic availability) of enterally administered BZDs (Hennessy, 1993; Lanusse & Prichard, 1993). These factors must be considered even when the anthelmintic drug

Pharmacokinetic parameters	TCBZ control	TCBZ + MTZ	TCBZ + PB	TCBZ + KTZ
$t_{½for}$ (h)	7.39 ± 0.77	8.12 ± 2.46	9.10 ± 1.66	8.31 ± 2.46
Cmax (μg/mL)	7.02 ± 1.93	5.50 ± 0.65	11.4 ± 1.92**	8.32 ± 1.86
Tmax (h)	21.6 ± 5.37	21.6 ± 10.0	30.0 ± 6.93	26.4 ± 13.2
$t½\beta$ (h)	24.3 ± 5.22	19.1 ± 5.56	23.5 ± 6.76	24.7 ± 10.1
AUC $_{0–144\ h}$ (μg·h/mL)	420.1 ± 103.6	309.1 ± 107.3	643.0 ± 151.2*	517.6 ± 166.4
MRT (h)	43.9 ± 7.93	42.5 ± 13.9	41.3 ± 8.33	46.7 ± 17.4

Table 3. Comparative pharmacokinetic parameters for triclabendazole sulphone ($TCBZSO_2$) obtained after administration of triclabendazole (TCBZ) parent drug (5 mg/kg) either alone or co-administered with the metabolic inhibitors methimazole (MTZ), piperonyl butoxide (PB) and ketoconazole (KTZ)

Data are expressed as mean ± SD (n = 5 animals).
Cmax, peak plasma concentration; Tmax, time to peak plasma concentration; $t_{½for}$, metabolite formation half-life; $t½\beta$, elimination half-life; AUC $_{0–144\ h}$, area under concentration-time curve from time 0 to 144 h after drug treatment; MRT, mean residence time. Values are significantly different from the TCBZ alone treatment at (*) $P < 0.05$a and $P < 0.01$.

under study is co-administered with a given metabolic inhibitor. Thus, selection of the IV route avoided the influence of those factors affecting the absorption of TCBZ and, consequently, the hypothetic metabolic interferences under study in the current work.

All metabolic inhibitors tested in this study were able to decrease the *in vitro* hepatic biotransformation of TCBZ (Virkel *et al.*, 2006). Although MTZ inhibited both TCBZSO and $TCBZSO_2$ formation *in vitro*, this FMO inhibitor did not induce noticeable changes on the kinetic disposition of TCBZ metabolites in sheep. A ~76% increment on TCBZSO $t½_{for}$ (see Table 2) was evidenced after the co-administration of TCBZ with MTZ in the current work, being the unique pharmacokinetic modification observed in the presence of this metabolic inhibitor. This observation may indicate a delayed *in vivo* FMO-mediated TCBZ sulphoxidation in the presence of MTZ, which did not induce changes on the plasma availability of TCBZSO. A clinically relevant drug interaction after the concomitant administration of two different compounds may occur if adequate concentrations are achieved at the site of action at the same time. Although there is not information available on the kinetic disposition of MTZ in ruminants, short half-lives characterised its plasma disposition kinetics in other species such as dogs (Vail *et al.*, 1994), cats (Hoffman *et al.*, 2002) and humans (Hengstmann & Hohn, 1985; Jansson *et al.*, 1985). The possible faster MTZ disposition after its IV administration in the current trial (opposed to the high concentrations of TCBZ achieved in the liver parenchyma) may have accounted for the lack of an *in vivo* metabolic interaction between both compounds under the experimental conditions described here. Speculations other than that above mentioned may be inconsistent, since MTZ induced pronounced changes on the disposition kinetics of other commonly used BZD anthelmintics (Lanusse & Prichard, 1993).

Co-administration of TCBZ with PB enhanced the plasma availabilities (measured as AUC) of TCBZSO (99%) and $TCBZSO_2$ (53%). These results are consistent with previous findings on the effects of PB on the kinetic disposition and clinical efficacy of fenbendazole and its sulphoxide derivative oxfendazole (Benchaoui & McKellar, 1996; Sánchez *et al.*, 2002). PB is a known inhibitor of the P450-mediated oxidation of TCBZ *in vitro* (Virkel *et al.*, 2006). Thus, the delayed TCBZ sulphoxidation and sulphonation may extend the time of TCBZSO and $TCBZSO_2$ formation, which would explain the increased availability of both metabolites in TCBZ + PB treated sheep. In addition, the reduced rate of the sulphonation reaction increased the proportion of the active TCBZSO metabolite measured in plasma up to 36 h post-treatment (see Fig. 4). Undoubtedly, inhibition of P450-mediated TCBZ oxidation accounted for the increased systemic availabilities of both TCBZ metabolites in sheep.

Ketoconazole, a broad spectrum antifungal agent, has been shown to be a selective inhibitor of P450 3A-mediated reactions (Newton *et al.*, 1995). Moreover, it has been shown that KTZ may also inhibit P450 1A2, 2D6 and 2C9 activities in human liver microsomes (Newton *et al.*, 1995), whilst its N-deacetyl metabolite, formed in a P450 3A-mediated reaction, undergoes FMO1 and FMO3-dependent N-oxidation (Rodriguez *et al.*, 1999). Inhibition of albendazole oxidative processes by co-administration of the N-substituted imidazoles, KTZ or clotrimazole, enhanced the plasma availabilities of albendazole sulphoxide and sulphone in rats (Merino *et al.*, 2003a,b). Inhibition of the hepatic P450-mediated $TCBZSO_2$ production by KTZ (Virkel *et al.*, 2006) agrees well with the increased AUC (41%) and Cmax (37%) values of TCBZSO when TCBZ was co-administered with the antifungal drug. The increased TCBZSO/$TCBZSO_2$ $AUC_{(0–36\ h)}$ ratio may also indicate inhibition of P450-mediated sulphonation following TCBZ + KTZ treatment.

From a mechanistic point of view (which could be clearly addressed in an *in vitro* experiment), inhibition of TCBZ oxidative metabolism *in vivo* should increase the plasma concentrations of the parent drug and decrease the P450-mediated formation of its sulphoxide and sulphone metabolites. However, the rapid depletion of TCBZ from the bloodstream, after a few hours of its intravenous administration, may accounted for the absence of noticeable changes on the plasma disposition kinetics of the parent drug after its co-administration with the P450 inhibitors tested. As high concentrations of TCBZ may be accumulated in the liver tissue, the metabolic interactions after the co-administration of the P450 inhibitors permit to observe the differences in the plasma concentrations of TCBZ metabolites.

Finally, this pharmacokinetic study was designed to establish whether or not metabolic interactions observed *in vitro* might be reproduced under *in vivo* conditions. Although MTZ inhibited both TCBZSO and $TCBZSO_2$ formation *in vitro*, this FMO inhibitor did not affect the kinetic disposition of TCBZ metabolites in sheep, presumably due to its fast elimination from the body after the IV administration. PB and KTZ inhibited the P450-mediated oxidation of TCBZ in the liver (and possibly in extra-hepatic tissues), which was reflected by an increased systemic availability of its active flukicidal TCBZSO metabolite. Moreover, co-administration of TCBZ with PB increased the availability of $TCBZSO_2$. This metabolite also has some flukicidal activity and may contribute to the overall TCBZ efficacy against *Fasciola hepatica* (Büscher *et al.*, 1999; Fairweather *et al.*, 2007).

No major new anthelmintic compounds have become available since the introduction of macrocyclic lactones endectocides in the veterinary market. Therefore, new pharmacological strategies are needed to improve the performance of existing anthelmintics. Modulation of drug metabolism in the host, as well as within the target parasites, should be considered among other available tools to optimise the efficacy of commonly used anthelmintic drugs in ruminants.

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REFERENCES

Alvarez, L., Sánchez, S. & Lanusse, C. (1999) *In vivo* and *ex vivo* uptake of albendazole and its sulphoxide metabolite by cestode parasites: relationship with their kinetic behaviour in sheep. *Journal of Veterinary Pharmacology and Therapeutics*, **22,** 77–86.

Alvarez, L., Imperiale, F., Sánchez, S., Murno, G. & Lanusse, C. (2000) Uptake of albendazole sulphoxide by *Haemonchus contortus* and *Fasciola hepatica* in sheep. *Veterinary Parasitology*, **94,** 75–89.

Alvarez, L., Mottier, L. & Lanusse, C. (2004) Comparative assessment of the access of albendazole, fenbendazole and triclabendazole to *Fasciola hepatica*: effect of bile in the incubation medium. *Parasitology*, **128,** 73–81.

Benchaoui, H. & McKellar, A. (1996) Interaction between fenbendazole and piperonyl butoxide: pharmacokinetic and pharmacodynamic implications. *Journal of Pharmacy and Pharmacology*, **48,** 753–759.

Bogan, J., Kinabo, L., Strong, M., Formica, C., Galtier, P. & Alvinerie, M. (1988) Pharmacokinetics of triclabendazole in cattle, sheep, goats, horses, ponies, donkeys, pigs and man. *Proceedings of the 4th Congress of the European Association for Veterinary Pharmacology and Toxicology*, 159–163, Budapest, Hungary.

Boray, J., Crowfoot, P., Strong, M., Allison, J., Schellenbaum, M., Von Ornelli, M. & Sarasin, G. (1983) Treatment of immature and mature *Fasciola hepatica* infections in sheep with triclabendazole. *Veterinary Record*, **113,** 315–317.

Brennan, G., Fairweather, I., Trudgett, A., Hoey, E., McCoy, M., McConville, M., Meaney, M., Robinson, M., McFerran, N., Ryan, L., Lanusse, C., Mottier, L., Alvarez, L., Solana, H., Virkel, G. & Brophy, P. (2007) Understanding triclabendazole resistance. *Experimental and Molecular Pathology*, **82,** 104–109.

Büscher, G., Bowen, F., Strong, M. & Crowfoot, P. (1999) Efficacy of triclabendazole, its metabolites and other compounds in sheep. In *Proceedings of the International Conference of the World Association for the Advancement of Veterinary Parasitology*, abstract c.7.40.

Cristofol, C., Virkel, G., Alvarez, L., Sánchez, S., Arboix, M. & Lanusse, C. (2001) Albendazole sulphoxide enantiomeric ratios in plasma and target tissues after intravenous administration of ricobendazole to cattle. *Journal of Veterinary Pharmacology and Therapeutics*, **24,** 117–124.

Fairweather, I. (2005) Triclabendazole: new skills to unravel an old(ish) enigma. *Journal of Helmintology*, **79,** 227–234.

Fairweather, I., Brennan, G., Shaw, E., Shaw, L., Halferty, L. & Toner, E. (2007). Relative activity of triclabendazole metabolites against the liver fluke *Fasciola hepatica*. In *Proceedings of the International Conference of the World Association for the Advancement of Veterinary Parasitology*, abstract 121.

Gibaldi, M. & Perrier, D. (1982) *Pharmacokinetics*, 2nd edn. Marcel Dekker, NewYork, USA.

Gottschall, D., Theodorides, V. & Wang, R. (1990) The metabolism of benzimidazole anthelmintics. *Parasitology Today*, **6,** 118–124.

Hengstmann, J. & Hohn, H. (1985) Pharmacokinetics of methimazole in humans. *Klinische Wochenschrift*, **63,** 1212–1217.

Hennessy, D. (1993) Pharmacokinetic disposition of benzimidazole drugs in the ruminant gastrointestinal tract. *Parasitology Today*, **9,** 329–333.

Hennessy, D., Lacey, E., Steel, J. & Prichard, R. (1987) The kinetics of triclabendazole disposition in sheep. *Journal of Veterinary Pharmacology and Therapeutics*, **10,** 64–72.

Hennessy, D., Steel, J., Lacey, E., Eagleson, G. & Prichard, R. (1989) The disposition of albendazole in sheep. *Journal of Veterinary Pharmacology and Therapeutics*, **12,** 421–429.

Hennessy, D., Steel, J., Prichard, R. & Lacey, F. (1992) The effect of co-administration of parbendazole on the disposition of oxfendazole in sheep. *Journal of Veterinary Pharmacology and Therapeutics*, **15,** 10–18.

Hennessy, D., Prichard, R. & Steel, J. (1993) Biliary secretion and enterohepatic recycling of fenbendazole metabolites in sheep. *Journal of Veterinary Pharmacology and Therapeutics*, **16,** 132–140.

Hoffman, S., Yoder, A. & Trepanier, L. (2002) Bioavailability of transdermal methimazole in a pluronic lecithin organogel (PLO) in healthy cats. *Journal of Veterinary Pharmacology and Therapeutics*, **25,** 189–193.

Jansson, R., Lindström, B. & Dahlberg, P. (1985) Pharmacokinetic properties and bioavailability of methimazole. *Clinical Pharmacokinetics*, **10,** 443–450.

Lanusse, C. & Prichard, R. (1991) Enhancement of the plasma concentration of albendazole sulphoxide in sheep following coadministration of parenteral netobimin and liver oxidase inhibitors. *Research in Veterinary Science*, **51,** 306–312.

Lanusse, C. & Prichard, R. (1992) Methimazole increases the plasma concentrations of the albendazole metabolites of netobimin in sheep. *Biopharmaceutics and Drugs Disposition*, **13,** 95–103.

Lanusse, C. & Prichard, R. (1993) Clinical pharmacokinetics and metabolism of benzimidazole anthelmintics in ruminants. *Drug Metabolism Reviews*, **25,** 235–279.

Lanusse, C., Gascon, L. & Prichard, R. (1995) Influence of the antithyroid compound methimazole on the plasma disposition of fenbendazole and oxfendazole in sheep. *Research in Veterinary Science*, **58,** 222–226.

Lubega, G. & Prichard, R. (1991) Interaction of benzimidazole anthelmintics with *Haemonchus contortus* tubulin: binding affinity and anthelmintic efficacy. *Experimental Parasitology*, **73,** 203–209.

Merino, G., Molina, A., García, J., Pulido, M., Prieto, J. & Alvarez, A. (2003a) Intestinal elimination of albendazole sulfoxide: pharmacokinetic effects of inhibitors. *International Journal of Pharmaceutics*, **263,** 123–132.

Merino, G., Molina, A., García, J., Pulido, M., Prieto, J. & Alvarez, A. (2003b) Effect of clotrimazole on microsomal metabolism and pharmacokinetics of albendazole. *The Journal of Pharmacy and Pharmacology*, **55,** 757–764.

Newton, D., Wang, R. & Lu, A. (1995) Cytochrome P450 inhibitors: evaluation of specificities in the *in vitro* metabolism of therapeutic agents by human liver microsomes. *Drug Metabolism and Disposition*, **23,** 154–158.

Notari, R. (1987) *Biopharmaceutics and Clinical Pharmacokinetics*, 4th edn. Marcel Dekker, New York, USA.

Perrier, D. & Mayersohn, M. (1982) Non-compartmental determination of the steady-state volume of distribution for any mode of administration. *Journal of Pharmaceutical Sciences*, **71,** 372–373.

Rodriguez, R., Proteau, P., Marquez, B., Hetherington, C., Buckholz, C. & O'Connell, K. (1999) Flavin-containing monooxygenase-mediated metabolism of N-deacetyl ketoconazole by rat hepatic microsomes. *Drug Metabolism and Disposition*, **27,** 880–886.

Sánchez, S., Small, J., Jones, D. & McKellar, Q. (2002) Plasma achiral and chiral pharmacokinetic behaviour of intravenous oxfendazole co-administered with piperonyl butoxide in sheep. *Journal of Veterinary Pharmacology and Therapeutics*, **25,** 7–13.

Vail, D., Elfarra, A., Panciera, D. & Hutson, P. (1994) Pharmacokinetics and short-term clinicopathologic changes after intravenous administration of a high dose of methimazole in dogs. *American Journal of Veterinary Research*, **55,** 1597–1601.

Virkel, G., Lifschitz, A., Sallovitz, J., Pis, A. & Lanusse, C. (2006) Assessment of the main metabolism pathways for the flukicidal compound triclabendazole in sheep. *Journal of Veterinary Pharmacology and Therapeutics*, **29,** 213–223.